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"Use of Second Messenger Assays to Detect Compound Efficacy"

medium above the monolayer of cells. The transfected cells are then incubated for 24 hours at 37° C in an atmosphere of 5-7% CO_2 . The medium and precipitate are then removed by aspiration, the cell layer is washed once with phosphate-buffered saline and 5ml of pre-warmed complete growth medium is added prior to incubating the cells for a further 24 hours at 37° C. The cells are then replated in the appropriate selective medium for the isolation of stable transformants. *Molecular Cloning, A Laboratory Manual: Vol 3, 16.30*, Editors Sambrook, Fritsch and Maniatis, 1989, a standard reference work in the art, sets out detailed explanations of this and other methods of transfection.

B. Confirmation of Constitutively Activated Receptors With Phosphorylation Assay

Receptor phosphorylation will be used as a first means of measuring constitutive activity in the expression system because only activated receptors are substrates for receptor kinases. This method has been used to measure the activated state of β-adrenergic receptors (Samama et al. 1993). Briefly, purified receptor preparations are incubated at 30°C for 20 min with β-adrenergic receptors (30 nM) in 20 mM Tris.HCl, pH 8.0, 2 mM EDTA, 10 mM MgCl, 1 mM dithiothreitol, 100 μM [γ-^{32}P]ATP (~2000 cpm/pmol), in the absence and presence of the relevant drugs (for orphan receptors the difference in basal phosphorylation level between constitutively active and non-active receptor will be used). At the appropriate times the reactions are stopped with an equal volume of 2X SDS sample-loading buffer (8% SDS, 25 mM Tris.HCl, pH 6.5, 10% glycerol, 5% mercaptoethanol, and 0.005% bromophenol blue) and are then electrophoresed on 10% SDS-polyacrylamide gels. Phosphorylation stoichiometries are then determined by excising and counting the receptor bands by using a phosphoImager.

III. USE OF SECOND MESSENGER ASSAYS TO DETECT COMPOUND EFFICACY

Once expression and activity of the constitutively activated receptors have been

confirmed, in order to study the compound efficacy of candidate compounds on the receptor, a method to detect the receptor-directed cellular response is most prefered. Several assay methods well known to those skilled in the art may be used with little modification. Because the invention disclosed herein allows the skilled artisan to screen large numbers of candidate compounds (e.g., chemical libraries) against the constitutively activated receptors, assay methods which lend themselves to high throughput screening are particularly advantageous. Examples of different assay methods that will be used are set forth below. These examples should be considered illustrative (and not limiting) of the type of assays which can be employed in the method of this invention. Different receptor types require different assays, and for any given receptor, one assay may be more appropriate than another. These choices are readily determined without undue experimentation and are well appreciated and understood by one skilled in the art.

A. G Protein-Coupled Receptors

1. GTP Membrane Binding & GTPase activity

It has been reported that [^{35}S]GTPγS can be used to monitor G-protein coupling to membranes in the absence and presence of ligand (Traynor and Nahorski, 1995). This assay is performed on membranes which have the expressed receptor. Briefly, membranes (100-200 μg of protein) are incubated with binding buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, and 10 mM MgCl.6$H_2$0) containing [^{35}S]GTPγS (80 pM), GDP (3 μM), and the small molecule drug (10-20 μM). The reported volume for performing this assay was 1 ml, but volumes ranging from 0.3-1 ml are useful. This is incubated for 60 min at 30°C. Bound and free [^{35}S]GTPγS can then be separated by vacuum filtration through GF/B filters and quantified by liquid scintillation counting. Inverse agonists are detected by their ability to reduce the scintillation signal.

a. GTP membrane binding for a scintillation proximity assay

A high-throughput screening assay can be used to measure the GTP binding to receptors using a scintillation proximity assay. Flash plates or scintistrips can be used which have scintillant coated on the wells. The binding assay can be performed as described above. However, when the membranes are centrifuged to the bottom of the wells, there is preferably need for separation of bound and "free" [^{35}S]GTPγS because the bound [^{35}S]GTPγS on the third intracellular loop will be in close proximity to the scintillant which will result in a signal.

Another way of performing the assay is to immobilize the membranes containing the expressed receptor onto flash plates. Briefly, the plates are coated with polylysine. The positively charged polylysine binds to the negatively charged phosphate groups and immobilizes the membranes. Any bound [^{35}S]GTPγS is then in close proximity to the scintillant and activates the scintillant, producing a signal. The assay can then be used to detect chemical compounds which inhibit this signal, which compounds are inverse agonists.

Another way of performing the assay will be to immobilize the membranes using Con A. Most seven transmembrane receptors contain sugar groups expressed on the extracellular regions of the receptor. Con A will bind to the sugar groups expressed on the extracellular side of the membrane and immobilize the membranes. The bound [^{35}S]GTPγS can then be detected by scintillation proximity as before.

b. GTPase activity

GTPase activity can also be measured by incubating the membranes containing the expressed receptor with [γ^{32}P]GTP. The released [γ^{32}P]Pi is then detected in the supernatant by scintillation counting (Hasegawa et al. 1996). Inverse agonists are detected by their ability to reduce the signal and agonists by their ability to increase the signal.

2. Adenylate Cyclase and Cyclic AMP (cAMP) Assays

Adenylate cyclase assays can be used to assess the efficacy of drugs interacting with constitutively active receptors. Briefly, the reaction medium contains phosphocreatine 5mM; creatine kinase 10 U/ml; BSA 0.04%; Tris HCl 50 mM Ph 7.4; $MgCl_2$ 5 mM; EDTA 0.25 mM; RO20-1724 0.12 mM; ATP 0.1 mM; GTP 0.1 mM [α-^{32}P]ATP between 1.5 and 2.5 μCi/tube; sucrose final concentration 65 mM. Assays are initiated by the addition of membrane suspension (~50 to 75 μg protein/tube) and the assay mixture incubated at 31°C for 1 h. (see Maenhaut et al. 1990 & Salamon et al 1974). Intracellular and extracellular cAMP can be determined using a standard cAMP radioimmunoassay or by using cAMP binding protein according to standard art methods.

3. Whole Cell Second Messenger Reporter Systems

Promoters on genes drive the expression of the proteins which that particular gene codes for. Cyclic AMP drives gene expression by promoting the binding of a cAMP-responsive DNA binding protein or transcription factor (CREB) which then binds to the promoter at specific sites called cAMP response elements and drives the expression of the gene. Reporter systems will be constructed which have a promoter containing multiple cAMP response elements before the reporter gene which can be β-galactosidase or luciferase, for example. Thus, an activated receptor causes the accumulation of cAMP (if it is coupled to a stimulatory G-protein) which then activates the gene and expression of the reporter protein. The reporter protein such as β-galactosidase or luciferase can then be detected using standard biochemical assays (Chen et al. 1995). Thus, a stable cell line will be constructed which has the cAMP-reporter construct. The activated receptor will then be transfected into this cell line which will activate the reporter gene expression. This assay system will then be used in the presence of small molecules from a chemical library to find compounds which inhibit the

reporter gene expression by interacting with the receptor; such inhibitors are inverse agonists identified by the method of this invention.

4. Intracellular Calcium Detection Using Calcium Sensitive Dyes

The activation of receptors on the cell surface often leads to an increase in intracellular "free" calcium levels. This increase in intracellular calcium can be detected in whole cell assays using calcium-sensitive dyes such as fura 2. A decrease in this signal caused by a chemical compound indicates inverse agonist activity.

5. Immediate Early Gene Induction

Early gene induction and expression is a downstream event of receptor activation. Activation and expression of transcription factors such as c-fos and CREB will be used as a means to monitor receptor activation and inhibition by drugs. The expressed CREB and c-fos can be detected by anti CREB and c-fos antibodies. These antibodies can be further detected by standard methods (e.g. anti rabbit horse-radish peroxidase antibodies with conventional peroxidase detection methods), with a decrease in the signal caused by a chemical compound indicating inverse agonist activity.

6. Cell Proliferation

Activation of G protein coupled receptors and tyrosine kinase receptors often results in cell proliferation. The ability of the cell to proliferate can be measured by the uptake of [^{3}H] thymidine into the DNA. Chemical compounds which block this proliferation can then be detected by a decrease in thymidine incorporation. The incorporation of bromo-deoxyuridine (BRDU) into the DNA can also be used as a measure of cell proliferation and receptor activation. The incorporated BRDU can be detected by scintillation counting or by anti-BRDU antibodies according to well known methods to determine compound-induced effects.

B. Tyrosine Kinase Receptors

Whole-cell second messenger reporter systems and intracellular calcium detection using calcium-sensitive dyes as described in sections C.1.c., C.1.d., and C.1.e. above will also be used to assay the tyrosine kinase activity.

IV. SCREENING OF CANDIDATE COMPOUNDS WITH CONSTITUTIVELY ACTIVATED RECEPTOR SYSTEMS

The method of this invention is well suited to modern techniques of high throughput screening (HTS) of, most preferably, large chemical libraries. Generally, when searching for potential drug candidates, what is desired is a compound which will have a high bioavailability. Recent advances in combinatorial chemical synthesis have produced large libraries of small organic compounds which have the potential to meet the bioavailability requirements. Since, once the method of this invention is known and appreciated, the technology exists for producing and assaying large numbers of constitutively activated receptor expression systems, high throughput screening of, for example, such chemical libraries or peptide libraries (e.g., in 96 well plate format) is straightforward. Therefore, application of the method of this invention is very useful as a straightforward manner to discover useful therapeutic compounds. A few examples of how such assays and screening are carried out illustrate the general approach, but are not intended to limit the scope of this invention.

A. Scintillation proximity assay (SPA) for cAMP

Constitutively active G-protein coupled receptors will be screened in a high throughput manner by measuring the formation of cAMP by scintillation proximity assay. One way of achieving this is to link an anti-CAMP antibody to scintillation proximity beads which are coated with scintillant. Thus, when radiolabeled CAMP is bound by the anti-cAMP antibody which is attached to the bead it comes into close proximity with the scintillant and activates